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Dental pulp stem cells: a novel cell therapy for retinal and central nervous system repair

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Abstract

Dental pulp stem cells (DPSC) are neural crest-derived ecto-mesenchymal stem cells that can relatively easily and non-invasively be isolated from the dental pulp of extracted postnatal and adult teeth. Accumulating evidence suggests that DPSC have great promise as a cellular therapy for central nervous system (CNS) and retinal injury and disease. The mode of action by which DPSC confer therapeutic benefit may comprise multiple pathways, in particular, paracrine-mediated processes which involve a wide array of secreted trophic factors and is increasingly regarded as the principal predominant mechanism. In this concise review, we present the current evidence for the use of DPSC to repair CNS damage, including recent findings on retinal ganglion cell neuroprotection and regeneration in optic nerve injury and glaucoma.

Abbreviations: DPSC, dental pulp stem cells; CNS, central nervous system; MSC, mesenchymal stem cells; GFAP, glial fibrillary acidic protein; SHED, stem cells from human exfoliated deciduous teeth; SCAP, stem cells from the apical papilla; PDLSC, periodontal ligament stem cells; NTF, neurotrophic factors; RGC, retinal ganglion cells; AMD, age-related macular degeneration; EGF, epidermal growth factor; FGF, fibroblast growth factor; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; GDNF, glial cell line-derived neurotrophic factor; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; BMSC, bone marrow-derived mesenchymal stem cells; AMSC, adipose-derived mesenchymal stem cells; RGC, TBI, traumatic brain injury; AD, Alzheimer's disease; PD, Parkinson's disease; SHH, sonic hedgehog; SCI, spinal cord injury; RPE, retinal pigment epithelium; TON, traumatic optic neuropathy

Introduction

Dental pulp, the vital inner core of teeth is a fibrous tissue containing mesenchymal stem/stromal cells (MSC) derived from the embryonic cranial neural crest [1]. Dental pulp stem cells (DPSC) are self-renewing and multipotent cells that express both MSC-like (e.g. CD29, CD90, CD105, CD146, CD166 and CD271) and neural stem cell-like (e.g. nestin, glial fibrillary acidic protein (GFAP)) phenotypic stem cell markers while being negative for hematopoietic markers such as CD45 [2-5]. DPSC can be readily isolated from the pulp of the 3rd adult molars (wisdom teeth) and expanded and stored for future use [2-4]. Dental stem cells can also be isolated from other discreet regions of the tooth including the dental follicle which surrounds developing teeth, termed dental follicle stem cells [6], the deciduous teeth of infants, termed SHED (stem cells from human exfoliated deciduous teeth) cells [7, 8], the apical papilla of immature teeth, termed SCAP (stem cells from the apical papilla) [9] and periodontal ligament (PDLSC) [10]. Regardless of origin, dental stem cells have shown rapid proliferation rates and are able to differentiate along typical mesodermal cell lineages such as chondrogenic, adipogenic and osteogenic lineages [5-11]. Their neural crest lineage, expression of neuronal markers and neurotrophic factors (NTF) as well as their potential neurogenic differentiation capabilities have driven research into assessing their potential use to treat neuronal disease and injury [3, 5, 12].

The CNS encompasses the brain and spinal cord and acts as the centre of all sensory perception and motor output. The CNS has very limited capacity for repair and regeneration; thus injury to this region is severely and permanently debilitating as damaged/axotomised neurons undergo cell death and are not replaced and most transected CNS axons do not regenerate [13]. Degeneration of injured CNS axons is attributed to the cessation of retrograde axonal transport of pro-survival NTF from the previously innervated targets. Functional loss can also be attributed to the loss of glia, particularly myelinating oligodendrocytes; however in most cases their loss is secondary to the damage/loss of

neurons. The retina is part of the CNS and suffers from the same limited capacity for repair. Injury to the retina can take multiple forms from photoreceptor/retinal ganglion cell (RGC) loss (AMD (age-related macular degeneration), glaucoma) to axonal transection (traumatic optic neuropathy) [13]. Stem cells may offer a suitable therapeutic approach to repair CNS, either by acting as a source of new neurons to integrate into neuronal tissue and replace those that have been lost (cell replacement), or by acting as a source of trophic factors to promote regeneration and survival of endogenous neurons (paracrine-mediated therapy; Figure 1). Although neural stem cells have been identified in the postnatal and adult brain, their role in endogenous functional CNS repair appears limited and isolation and amplification of these cells is met with various technical hurdles and ethical concerns. Embryonic and induced-pluripotent stem cells have also demonstrated potential, particularly for cell replacement, with the latter overcoming many of the ethical concerns affecting the former. However, new emerging therapies utilizing postnatal or adult MSCs have gained increasing attention demonstrating promising potential for neural tissue repair and regeneration, in particular through stimulation of endogenous repair processes by secreted paracrine factors [14-16].

In the following concise review we focus on the therapeutic potential of DPSC (also regarded an ecto-MSC due to its neural crest origin), and where relevant, in comparison to other more widely used MSC types to underscore the potential advantage of DPSC as a neuroprotective and neuroregenerative cell therapy for patients after CNS trauma [16, 17]. We will briefly discuss the neurogenic and neurotrophic properties of DPSC, followed by summarising the application of DPSC for brain and spinal cord repair, and in conclusion our recent work on the use of DPSC for retinal neuronal regeneration.

The case for DPSC as neuronal cell therapy

Neurogenic differentiation potential of DPSC

The classical application for stem cells is based on their ability to proliferate and differentiate into new specialised cells to facilitate replacement and regeneration of tissues. In particular, the neural crest origin and nestin-expression of DPSC has supported the notion that these cells may be amenable to differentiation into functional neurons and hence are suitable as source of replacement cells for injured neuronal cells [1-4, 16]. DPSC have been reported to differentiate into neurons when treated with typical neurogenic supplements such as epidermal growth factor (EGF), basic fibroblast growth factor (FGF) and retinoic acid [18] as well as forskolin [19], expressing neuronal specific neurofilament medium- and heavy-chain peptides, generate sodium currents [18] as well as voltage-dependant sodium and potassium channels. Utilizing these factors, pre-differentiated DPSC survived and integrated into the brain parenchyma after transplantation into the cerebrospinal fluid of rats following a cortical lesion and continued to remain function for up to 4 weeks [20]. DPSC-derived neurons maintained their expression of mature neuronal markers (such as NeuN), voltage-gated sodium channels and delayed rectifier potassium channels [20]. However, a separate study contradicted these claims, suggesting that DPSC may differentiate into neuronal precursor cells based on expression of typical phenotypic markers, but was unable to fully differentiate into mature neurons lacking the ability to generate action potentials [21]. It should also be remarked that MSC/DSPC represent relative heterogeneous cell populations with the possibility that specific subsets of cells may display distinct differentiation profiles, including cell types with increased neurogenic capacity [22]. In conclusion, the identity of neurogenic MSC/DPSC and their differentiation potential needs further clarification. Although DPSC seem to lag behind the impressive advances seen with embryonic and induced pluripotent stem cell research (reviewed in [16]), enabling differentiation into not only functional but also specialised neurons including all those that make up the retina [23, 24], the therapeutic prospects of these dental stem cells as neurogenic support for nerve regeneration may be substantial.

Neurotrophic properties of DPSC

It is now widely recognised that the predominant therapeutic action by MSC is paracrine-mediated through the secretion of trophic and anti-inflammatory factors. Various studies have demonstrated the significant neurotrophic expression and secretion of DPSC encompassing nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), glial cell-line derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF) and platelet-derived neurotrophic factor (PDGF) [12, 25-27]. These NTF have varying degrees of efficacy and importance with NGF being predominantly axogenic/neuritogenic while BDNF and PDGF being more essential for neuroprotection [26]. Our recent work confirmed that a range of DPSC neurotrophic genes and factors, including NGF, BDNF, NT-3 and VEGF with GDNF and PDGF, exceeded the levels expressed and secreted by other MSC types such as bone marrow (BMSC) and adipose-derived MSC (AMSC) [25, 26]. Along with driving axon regeneration, DPSC derived factors have also been reported to mediate axon guidance, shown elegantly following transplantation into a developing chick embryo. Although these results were done in the trigeminal nerve, a component of the peripheral rather than CNS, authors demonstrated that DPSC-derived CXCL12 was an important factor in guiding axons along the trajectory of axonal growth. These results suggest a potential for DPSC to promote and guide the regeneration of injured CNS axons to their necessary targets [28]. In contrast to these fascinating findings, embryonic and induced pluripotent stem cells lack evidence for any significant paracrine support [16]. Therefore, MSC and particular DPSC represent an ideal cell type for indirect repair and protection of CNS injury sites

DPSC for repair of brain injury: potential treatment for TBI/Stroke

An effective stem cell-based treatment for brain injuries such as traumatic brain injury (TBI) and stroke would require cells that adequately graft, integrate and remain within the brain. DPSC transplanted into healthy uninjured brain stimulated migration and proliferation of

endogenous neural cells and also increases the expression of NTF such as ciliary neurotrophic factor, VEGF and FGF within the graft site [29]. Although the graft itself was short lived, these results underscore the potential of DPSC to modulate brain tissue indirectly through paracrine-mediated mechanisms.

Stroke is a life threatening cerebrovascular condition resulting in ischaemic damage to the brain and remains the second leading cause of death. Intracerebral transplantation of DPSC in a rodent model of focal cerebral ischemia led to an improvement in forelimb sensorimotor function, despite only 2% of transplanted DPSC migrating and engrafting into the lesion site 4 weeks after transplantation [30]. DPSC predominately differentiated into glia as identified by GFAP (glial fibrilliary acidic protein) expression rather than neurons (neuronal specific enolase marker), suggesting that the functional benefit elicited by DPSC was indirect, paracrine-mediated as opposed to directly replacing neurons that have been lost due to ischaemic damage [30]. The fact that the majority of DPSC-derived cells were rapidly cleared despite the improvement in function corroborates the theory that DPSC promote functional regeneration of endogenous CNS tissue. In another report, DPSC transplanted into the ventricles of animals with hypoxic-ischaemic brain damage promoted the survival and formation of neuronal and glial cells while improving functional performance as assessed by a variety of behavioural tests [31]. Although these results implied that that DPSC may have formed new cells to replace the damaged neurons, considering the low survival rate of the grafted cells, it is plausible to assume that the achieved neuroprotection was due to indirect endogenous stimulation and protection of host neurons and glia [31]. Using SHED cells in a model of hypoxic-ischaemic injury of the brain in mice, it was found that SHED transplantation improved neurological function as measured by behavioural foot-fault testing whilst preventing tissue atrophy and reducing the number of endogenous apoptotic cells [32]. Interestingly, differentiation into neuron and glia was reportedly absent, suggesting the neuroprotective benefits and functional improvement were caused by paracrine-mediated processes.

One study using DPSC pre-differentiated with EGF, bFGF and retinoic acid into neuron-like cells described that, after transplantation into the cerebrospinal fluid of rats with a TBI, DPSC-derived cells migrated into various brain regions including the lesion site and adopted a neuronal phenotype expressing functional sodium/potassium currents [20]. Taken together, we feel that these findings underscore the capability of DPSC to migrate and survive within a CNS lesion site offering a suitable therapy for TBI either through pre-differentiation and replacement of lost neurons or as paracrine-mediated supporters of endogenous neuronal survival and axonal sprouting vehicles.

DPSC have also been considered for neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's diseases (PD), which are characterised by the gradual and permanent loss of neurons. Using *in vitro* models of AD/PD, co-culture of DPSC with hippocampal and mesencephalic neurons treated with amyloid beta peptide or 6-hydroxydopamine (6-OHDA) significantly reduced toxicity and death of neurons, which could be related to the expression of several NTF including NGF, GDNF and BDNF by DPSC [33]. DPSC also appeared to differentiate into dopaminergic neurons *in vitro* after treatment with sonic hedgehog (SHH), FGF9, GDNF and forskolin, although whether they have the ability to maintain this phenotype and replace lost dopaminergic neurons in PD *in vivo* has not yet been established and warrants further research [34].

Potential DPSC treatment for SCI

Spinal cord injury (SCI) is a severe debilitating condition that results in permanent partial or complete loss of sensation, paralysis of lower extremities and in severe cases, upper extremities and respiratory arrest. In a seminal study, DPSC were tested for their efficacy as a treatment for SCI in comparison with BMSC and AMSC [12]. DPSC were shown to promote *in vitro* neuritogenesis over an inhibitory medium containing chondroitin sulphate

proteoglycans and myelin-associated glycoprotein, underlying their potential as a paracrine-based therapy for promoting axon regeneration through the surrounding inhibitory factors of the spinal cord lesion site. After transplantation into SCI sites, DPSC did not differentiate into neurons but did promote the survival of endogenous neurons and glia within and around the lesion site [12]. Remarkably, surviving corticospinal tract axons regenerated over significant distances across the lesion site and biotinylated dextran amine (BDA)-labelling which confirmed not only extensive axon regeneration into the scar of the lesion site but also regeneration beyond the epicentre and into the distal cord. The substantial axon regeneration by DPSC correlated with improved results in the functional hind-limb locomotory tests, and particularly noteworthy, was significantly more than in animals receiving BMSC or AMSC transplants. Although this study primarily supported DPSC therapeutic potential through paracrine-mediated mechanisms, oligodendrocyte differentiation and subsequent remyelination of regenerated axons may have indirectly contributed to the functional restitution in these animals, presenting a potentially secondary mechanism. Further studies have looked into the potential benefit of glia-derived DPSC by inducing them to differentiate into Schwann-like glial cells which secrete greater titres of NTF compared to undifferentiated DPSC. *In vitro*, these Schwann-like cells promoted greater regeneration of dorsal root ganglion cell (from the spinal cord) neurites compared to DPSC [35]. Corroborating these results, SHED cells, pre-differentiated down a neural lineage before transplantation into a SCI site also improved locomotion in rats after SCI, underlining the potential of dental stem cells as promising cell therapy for CNS repair and regeneration [36].

DPSC treatment for retinal repair in ocular injury or disease

The retina is a complex structure composed of three layers of interconnected neurons, photoreceptors, bipolar cells and RGC laying atop the retinal pigment epithelium (RPE) and also populated by supportive amacrine and horizontal cells. The retina and optic nerve,

formed during development as an outgrowth of the brain [13], is an integral part of the CNS. Ocular disease can arise from a degenerative chronic abnormality such as AMD characterised by a slow progressive loss of retinal photoreceptor and RPE cells. On the other hand, glaucoma entails degeneration of RGC due to compression of the optic nerve, typically (but not necessarily) due to elevated intraocular pressure. Traumatic optic neuropathy (TON) is rather analogous to SCI and often involves damage to the optic nerve leading to a sudden acute loss of retinal cells and their axons and an immediate loss of visual function.

Previous proof-of-principle studies have shown that primary photoreceptors transplanted into the eye integrate into the outer nuclear layer of the retina and restore visual function [32]. However obtaining large numbers of photoreceptors from young donor eyes is clinically not feasible [37, 38] and thus an alternative therapeutic strategy for AMD is needed, ideally a stem cell source which is easily accessible and can be expanded and differentiated into photoreceptor cells. The usefulness of DPSC as potential treatment for AMD is not yet known. It was reported recently that DPSC can be induced to differentiate into a photoreceptor phenotype after exposure to conditioned medium from injured organotypic retinal cultures [39]. These DPSC-derived photoreceptors express the phenotypic marker rhodopsin but their functional activity has not yet been confirmed.

DPSC have also recently been suggested to have the potential to differentiate into RGC-like cells [40]. When treated with FGF2 and SHH while seeded on a 3-dimensional fibrin hydrogel, DPSC showed phenotypic signs of RGC differentiation including the expression of RGC associated genes/proteins such as Brn3b; however, again it is still unclear whether these cultivated cells are bona fide RGC and functional that can be used to successfully integrate into the retina to restore vision. Another important consideration is that DPSC-derived RGC would need to regenerate an axon along the complete length of the optic nerve before it would be functional, which has not been achieved as yet [13]

Recent studies explored the possibility of MSC-mediated retinal repair through paracrine-mediated mechanisms [41]. RGC death is mostly instigated by the lack of retrograde supply of essential survival factors (i.e. NTF), and experimental treatments with recombinant NTF have demonstrated neuroprotective efficacy, albeit in a transient and short-lived fashion [13]. As MSC/DPSC have relatively high expression of a range of NTF, we started to investigate their RGC neuroprotective effects in co-cultures with axotomised, injured primary rat RGC in a transwell system whereby the two cell populations were separated by a semi-permeable membrane [25, 26]. DPSC promoted significant survival of cultured RGC and regeneration of their neurites (Fig. 2A, B), an effect that was largely dependent on NTF secretion since neuroprotection/pro-regeneration was abolished when specific fusion protein inhibitors to NTF-receptors are added to the cultures.

We next tested the paracrine-mediated benefits by transplanting them into the vitreous of animals following an optic nerve crush (see also Fig. 1). Direct delivery of stem cells has been shown to be required, particularly as the vitreous lies adjacent to the retina and thus MSC-derived trophic factors will be within the RGC microenvironment. Systemic delivery (into peripheral blood) of BMSC failing to migrate into the retina and providing no therapeutic benefit, as opposed to when delivered locally into the vitreous [41]. Following transplantation into the vitreous, DPSC survive for the 3 weeks and promote survival of approximately 40% of RGC (Fig. 2C, D) with a significant increase in both regeneration (Fig. 2E-H) of their axons and dissolution of scar within the lesion site [25]. The relatively pronounced longevity of transplanted intravitreal DPSC is similar to other MSC and is likely due to the immunoprivileged environment of the eye, immunosuppressive properties of the MSC and the constrained nature of the vitreous preventing cellular migration [16], lending support to the potential for DPSC/MSC to act as a long-term therapy. The neuroprotective and axogenic effects elicited by the transplanted DPSC are significantly more pronounced than after BMSC transplantation, further emphasising the potential of DPSC. Recently, we

investigated the potential of human DPSC in an animal model of glaucoma in which intraocular pressure is elevated leading to a slow progressive loss of RGC [42]. We delivered human-derived DPSC into the vitreous and recorded both the number of surviving RGC and their electrical activity by electroretinography, which is indicative of their function. DPSC not only protected RGC from death but also preserved visual function significantly compared to both untreated eyes and eyes treated with BMSC/AMSC for up to 35 days. Studies confirming their therapeutic efficacy at longer time points have yet to be conducted. This is the first time DPSC have been tested in a glaucomatous model and the demonstrated efficacy has important implications considering the ongoing clinical trials using BMSC to treat various ocular diseases [16].

Future work (and challenges)

Despite the recent and exciting advances in the field, challenges remain before DPSC are accepted as a clinical therapeutic for CNS disease. Still little is known about the precise mechanisms of action of DPSC and the role of the host environment in the endogenous repair in response to DPSC/MSC therapy. The MSC/DPSC secretome is a complex mixture of bioactive factors and further knowledge is needed as to which specific factors are key for therapeutic and other effects, including possible anti-inflammatory and angiogenic reactions. Thus further research may facilitate development of combinatorial stem cell-based therapies or cell-free approaches involving purified secretome fractions or exosomes. Moreover, although a large number of studies suggest that the mechanism is predominantly paracrine-mediated, a role of DPSC differentiation into neurons and glia cannot be fully excluded. Indeed the disparity between the successes of DPSC differentiation *in vitro* and lack thereof *in vivo* suggests that the host environment plays a significant role. Injured neurological tissue presents a vastly different environment to the carefully controlled *in vitro* setting and studies on what factors may be preventing *in vivo* differentiation may greatly improve the potential of cell replacement strategies. Equally, the limiting factor for successful differentiation may be a

lack of migration of transplanted DPSC to the injured microenvironment. DPSC migrate to areas of tooth injury to replace lost cells and identification of the specific chemokines and receptors responsible may provide a potential therapeutic adjunct to cell replacement therapies [43]. Indeed some factors have already been identified and include stromal cell-derived factor 1α, granulocyte-colony stimulating factor and FGF2 [43] with the former having shown efficacy *in vivo* [44].

For clinical translation, it is of paramount importance to address risk and safety issues as well as standardisation and optimisation of the isolation, culture and cryostorage conditions to meet regulatory requirements using DPSC isolated from extracted human teeth. The variability in NTF secretion by DPSC between different donors [12, 25, 26] may present a formidable problem to the standardisation process and cellular treatments may be required from a pool of mixed donors or as highlighted before, cell-free approaches utilising the MSC secretome, to attain a consistent therapeutic efficacy. An important question to address is what happens with the DPSC on a long-term basis following cell transplantation, i.e. what is the fate and distribution of the cells (within the vitreous of) the eye. Noteworthy, clinical trials have already begun to address safety issues for MSC transplantation in the eye [16]. Additional work should also include the development of suitable cell delivery systems such as using encapsulated cells within a semi-permeable biomaterial that will preserve the paracrine-mediated effects whilst limiting the risk of uncontrolled migration/proliferation [45, 46].

Another issue is the correct dosing of the stem cells to ensure maximum efficacy. For example, transplantation of BMSC into the vitreous of TON animals demonstrated that the extent of axon regeneration increases with increased dosing of the BMSC transplants [47]. Further work is also warranted to optimise NTF production by DPSC for example by altering the pre-culture conditions to prime and/or pre-differentiate the cells. Indeed, a recent study elegantly demonstrated that following pre-differentiation into Schwann-like glial cells, the

DPSC secreted significantly increased levels of NTF and were able to further stimulate neurite outgrowth in an *in vitro* dorsal root ganglion injury model of SCI as compared with non-differentiated cells [35].

Conclusions

DPSC possess great potential in the treatment of traumatic and degenerative neurological conditions, the paramount mechanism of action after transplantation is probably paracrine-mediated, with secreted NTF orchestrating sustained neuronal survival, axon regeneration and functional restoration and preservation. Cell differentiation may possibly play a role, in particular into glial-like cells which then may function either as a source of NTF or as supporting/remyelinating cells. The differentiation of DPSC into functional neurons is still contentious with the majority of studies restricted to *in vitro* scenarios whereas those transplanting DPSC-derived neural cells *in vivo* have yet to be definitively shown to replace and restore damaged neuronal circuits. DPSC have only been a focus of research for a relatively short period of time and as such, no clinical trials have been conducted to measure clinical efficacy. However, considering the substantial therapeutic potential of these cells, we anticipate a rapid increase in research into this area and predict that DPSC-based clinical trials will become reality in the not too far away future.

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Figure 1: Schematic diagram demonstrating the application of dental stem cells in neural retinal repair. Dental stem cells occupy several discrete region of the tooth and surrounding tissue, of which, DPSC are found within the adult pulp. Isolation of DPSC from extracted adult teeth via relatively easy and non-invasive procedures; subsequent culture of DPSC allows expansion and potential manipulation (e.g. stimulation or pre-differentiation by defined factors) prior to transplantation. Following transplantation, DPSC may act either by replacing lost neurons *via* integration and differentiation into the affected tissue (A) and/or, supporting/promoting the endogenous regeneration of injured tissue through the secretion of multifiunctional active diffusible growth factors (e.g.NTFs).

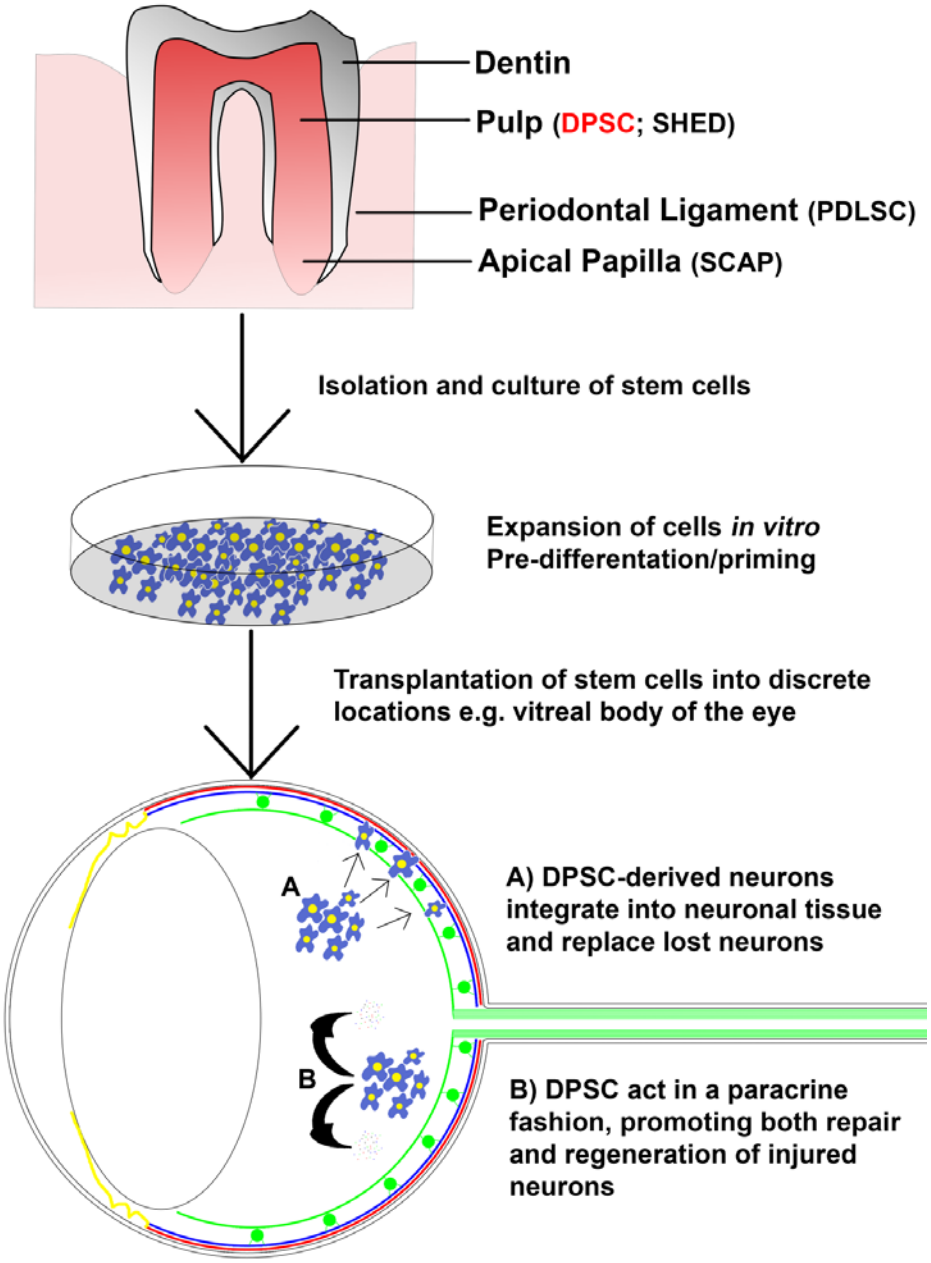

Figure 2: The paracrine-mediated effects of DPSC on injured CNS neurons (RGC).

A-B: Injured βIII-tubulin^{+} adult rat RGC co-cultured in a transwell with human DPSC (A) have significantly increased survival and regeneration of their neurites compared to co-culture with control cells (dead DPSC/fibroblasts (B).

C-H: Transplanted into the vitreous of rats, DPSC elicit a significant neuroprotective effect on Brn3a^{+} RGC (C) after optic nerne crush injury (ONC) and also strong activation in supportive Müller glia compared to animals receiving control cells (D). In the same animals, intravitreally transplanted DPSC promoted significant regeneration of GAP-43^{+} RGC axons in the optic nerve at both the crush site (E) and 2mm distal to the crush site (G) in compared to control animals (F, H). Scale bars represent 50µm (A-D), 100µm (E, F) and 200µm (G, H).

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